Bulletin UASVM Animal Science and Biotechnologies, 66 (1-2)/2009
Print ISSN 1843-5262; Electronic ISSN 1843-536X

Development of Innovative Molecular Methods for the Detection and the Identification of *Pseudomonas* spp. in Environmental and Clinical Samples

Cecilia CALISTI, Maurizio RUZZI

Dept. Agrobiology and Agrochemistry, University of Tuscia,
Via C. de Lellis, snc, I-01100 Viterbo, Italy; ruzzi@unitus.it

Abstract. We have developed a rapid, reliable and sensitive method to analyze, detect and trace dissemination of pathogenic and spoilage *Pseudomonas* species in foods and environments. The molecular identification of *Pseudomonas* species was achieved using a PCR-based assay with primer sets specific for 16S ribosomal DNA and *gyrB* genes. This PCR assay was found to provide highly genus-specific detection and could be successfully used to identify *Pseudomonas* in microbial consortia where these bacteria were not abundant. By coupling the PCR assay for 16S rDNA gene to a T-RFLP technique, identification of *Pseudomonas* strains at species level could be obtained without cultivation. PCR with a combination of two target sequences (multiplex PCR) appeared to be the optimum choice for discriminating between *Pseudomonas* and closely related genera.

Keywords: *Pseudomonas* species, *Pseudomonas aeruginosa*, molecular typing, PCR, T-RFLP, 16S ribosomal DNA, *gyrB* gene.

INTRODUCTION

Pseudomonas is one of the most diverse bacterial genus, comprising over sixty validly described species with multiple biovars (Anzai *et al.*, 2000). Members of this genus are aerobic or facultative anaerobic gram-negative chemo-organotrophs, able to grow in soil, marshes, and marine habitats, as well as on plant and animal tissues. This genus includes plant growth promoting species (Mark *et al.*, 2006; Weller, 2007) and some species that can be opportunistic pathogens to plants, humans, and animals (Lyczak *et al.*, 2000).

Identification of these species may be problematic due to the marked phenotypic variability demonstrated by isolates from clinical and environmental sources. The current strategy of bacterial identification is biochemical testing, consisting of primary detection with various standard culture media and identification using commercial identification systems, such as Vitek system (bioMerieux, Hazelwood, MO). However, these identification methods may misidentify fluorescent pseudomonads, such as *P. aeruginosa* (Qin *et al.*, 2003; Saiman *et al.*, 2003). Molecular method based on the detection of nucleic acids hold the promise the rapid detection and identification. Several PCR screenings, targeting several gene sequences (*toxA, gyrB, oprI, oprL, fliC, ecfX*) or the ribosomal operon, have been developed for identification and characterization of *P. aeruginosa* providing evidences that molecular techniques are superior over conventional phenotypic methods (Atzél. *et al.*, 2008).

The aim of the current study was to develop a rapid and sensitive culture-independent method for analysis of *Pseudomonas* populations in environmental and clinical samples, capable of distinguishing different fluorescent species and of identifying *P. aeruginosa* strains at species level.

MATERIALS AND METHODS

Total genomic DNA from pure cultured strains and environmental samples was extracted using DNeasy Blood & Tissue Kit (Qiagen) according to manufacturer's instructions. Experimental specificity of PCR primers targeting the 16S rDNA of *Pseudomonas* species was tested using genomic DNA from laboratory strains and DNA from environmental samples. PCR reactions were performed in a total volume of 50 µl containing 0.2 mM of each dNTP (Fermentas, Lithuania), 0.2 µM of each primer, 1 µl DNA template, 5 µl of 10x PCR buffer and 2.5 U of enzyme. A variety of Taq polymerases from a number of different suppliers were used successfully. These included Taq DNA polymerase from Qiagen and Biolabs, MasterAmp from Epicentre, MasterTaq from Eppendorf and AmpliTaq Gold from Perkin Elmer. Details of the primers and PCR conditions are available elsewhere (http://dspace.unitus.it/handle/2067/587). Negative controls without template DNA were included for each PCR experiment. PCR products were verified by electrophoresis in 1% (w/v) agarose gel stained with ethidium bromide.

Terminal Restriction Fragment Length Polymorphism (T-RFLP) analysis was performed on DNA from several environmental samples. These included DNA from bacterial populations from spent mushroom compost (SMC), nutrient solution from closed hydroponic systems (NS; Pagliaccia *et al.*, 2008) and a packed-bed reactor for aerobic biological treatment of olive mill wastewater (SB; Bertin *et al.*, 2006). Fluorescently labeled PCR products (100 ng) were purified and digested with endonuclease *Hha*I (Roche) and *Msp*I (Roche) in separate reactions. Each digestion was performed at 37°C for at least 4 h. Aliquots (1 µl) were mixed with 19,5 µl of deionized formamide and 0,5 µl of ROX-labeled GS500 internal size standard (Applied Biosystems). Genescan 3.1 software was used to quantify the electropherogram output by setting the peak height threshold of 100 fluorescent units. Replicate T-RF profiles gave reproducible fingerprints.

RESULTS AND DISCUSSION

The design of genus-specific primer pairs for the selective amplification of the 16S rRNA regions of the genus *Pseudomonas* were based on the search for conserved sequences within this genus. Three regions were found to be enough conserved among the different species of the genus and two genus-specific primer pairs were designed.

The specificity of the two primer pairs (named P1-P3 and P2-P3, respectively) was experimentally verified by PCR using genomic DNA from 62 pure cultures. The cultures analyzed included 41 *Pseudomonas* and 21 non-*Pseudomonas* strains. PCR assays employing each primer pair generated a DNA product of the expected size only when DNA extracted from *Pseudomonas* strains was used as a template (Table 1). This result indicated that the sensitivity and specificity of each PCR assay was 100%. The results were reproducible and comparable for all of the Taq DNA polymerases used in this work.

To further evaluate the specificity of the PCR protocol, metagenomic DNA extracted from three different environmental samples was used as a complex source for 16S rRNA gene amplification targets of culturable and nonculturable microorganisms. A product of the expected size was obtained from each metagenome. The accuracy of the amplification protocol was evaluated by restriction pattern analysis and sequencing of representative clones derived from the amplified region of the 16S rRNA gene. The results of these experiments are summarized in Table 2.

Tab. 1

Inclusivity and exclusivity data for the two 16S rDNA PCR assays

Bacterial species (N° of isolates tested)	PCR product	
	P1-P3 (1081 bp)	P2-P3 (860bp)
Pseudomonas aeruginosa (24)	+	+
Pseudomonas avellanae (1)	+	+
Pseudomonas chlororaphis (1)	+	+
Pseudomonas fluorescens (4)	+	+
Pseudomonas putida (4)	+	+
Pseudomonas oleovorans (1)	+	+
Pseudomonas savastanoi (4)	+	+
Pseudomonas syringae (1)	+	+
Pseudomonas stutzeri (1)	+	+
Acinetobacter baylyi (1)	−	−
Acinetobacter baumannii (1)	−	−
Aeromonas hydrophila (1)	−	−
Agrobacterium tumefaciens (1)	−	−
Bacillus subtilis (1)	−	−
Brenneria quercina (1)	−	−
Burkholderia terricola (1)	−	−
Clostridium spp. (1)	−	−
Enterococcus faecalis (1)	−	−
Erwinia amylovora (1)	−	−
Escherichia coli (1)	−	−
Halomonas spp. (1)	−	−
Marinomonas spp. (2)	−	−
Paracoccus denitrificans (1)	−	−
Pseudoalteromonas spp. (3)	−	−
Rhizobium spp. (1)	−	−
Salmonella typhimurium (1)	−	−
Stenotrophomonas maltophila (1)	−	−

Note: + presence, − absence

Tab. 2

Specificity of the PCR protocol on metagenomic DNA

Metagenome	Primer pair	T-RF lenght (bp)	Clone distribution (%)*
Spent mushroom compost (SMC)	P1-P3	398[1]/(>600) [2]/115[3]	*P. fluorescens* (100)
	P2-P3	233[1]/621[2]/307[3]	*P. fluorescens* (100)
Nutrient solution from closed hydroponic systems (NS;)	P1-P3	398[1]/552[2]/115[3]	*P. putida* (100)
	P2-P3	233[1]/387[2]/307[3]	*P. putida* (100)
SB reactor for the aerobic biological treatment of olive mill wastewater (SB)	P1-P3	398[1]/115[3]	*Pseudomonas* spp. (100)
	P2-P3	233[1]/(>500)[2]/307[3]	*Pseudomonas* spp. (60)
		234[1]/187[2]/111[3]	*Dokdonella* spp. (40)

Note: * The clone distribution was based on the sequence of 16S rRNA genes recovered.
[1] *Msp*I, [2] *Rsa*I, [3] *Hha*I

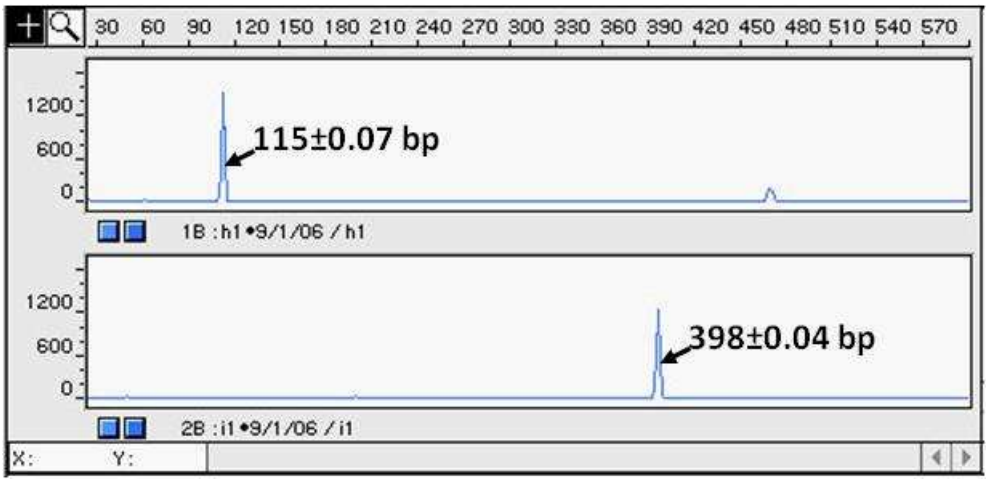

Fig. 1. T-RF profiles derived from enzyme digestion with *Hha*I (upper panel) or *Msp*I (lower panel) of amplicons from NS metagenome generated with primer pair P1-P3.

TRF patterns derived from enzyme digestion with *Rsa*I, *Hha*I and *Msp*I of amplicons generated with primer pair P1-P3 and P2-P3 revealed the presence of 16S rDNA sequences belonging to fluorescent *Pseudomonas* strains in all of the metagenomic DNA samples tested.

An example of TRF patterns produced after digestion of amplicons from NS metagenome is showed in Fig. 1.

Based on the T-RF lengths (Table 2), we could demonstrate that *P. fluorescens* strains are present in SMC and SB communities, while the NS community includes strains of *P. putida*. Sequence data from clone libraries of 16S rRNA genes generated from the same environmental samples were in agreement with T-RFLP data. Fingerprints from SB metagenome produced by *Hha*I and *Rsa*I digestion of amplicons generated with primer pair P2-P3 generated two different profiles, one of which does not match any the expected fingerprints (Table 2).

Comparative analysis of bacterial 16S rRNA gene sequences from SB clone library with the sequences present in the RDP database showed significant similarity with the 16S rDNA from *Dokdonella*, a gram-negative bacterium belonging to the family of Xhantomonadaceae. These data indicated that, when metagenomic DNA was used as a template, primer pair P2-P3 generated an amplicon of the expected size also with 16S rDNA of some non-*Pseudomonas* strains (i.e bacteria closely related to *Dokdonella* group) (Table 2). On the contrary, when primer pair P1-P3 was used, it was obtained an amplicon of the expected size only when 16S rDNA of *Pseudomonas* strains was present. Taken together, the data confirm the selectivity of the primer pair P1-P3, as well as of the PCR protocol for the identification of fluorescent *Pseudomonas* at genus and species level.

For accurate identification of *P. aeruginosa* we have also developed a Multiplex-T-RFLP assay. The molecular assay was based on the simultaneous amplification of two target sequences (16S rRNA and *gyrB* sequence) and on the analysis of the PCR products by T-RFLP. The assay was validated on total genomic DNA of 24 different strains of *P. aeruginosa* isolated from environmental and clinical samples. Both target genes used in the multiplex-PCR were detected and T-RFs of expected size were obtained from all *P. aeruginosa* strains tested, confirming the specificity and selectivity of this assay for the identification of *P. aeruginosa* isolates at species level.

CONCLUSIONS

We present here a significantly improved DNA fingerprinting assay for rapid and accurate identification of fluorescent *Pseudomonas*.

By coupling the PCR assay for 16S rDNA gene to a T-RFLP technique, identification of *Pseudomonas* strains at species level could be obtained without cultivation.

Simultaneous amplification of 16S rDNA and *gyrB* followed by digestion of amplicons, which are then separated by multicolor capillary electrophoresis, allowed rapid and accurate identification of *P. aeruginosa* isolates at species level.

Acknowledgments. This study has been financed by Italian National Institute for Occupational Safety and Prevention.

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